Influence of Two Mass-Marking Techniques on Survival and Flight Behavior of *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)

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ABSTRACT Two mass-marking techniques were evaluated in the laboratory to determine effects on survival and flight behavior of *Diabrotica virgifera virgifera* LeConte. The marking agents were an externally applied fluorescent powder (Day-Glo) and an internal oil soluble dye (Calico oil red) incorporated into an artificial adult diet. Neither technique had a significant influence on survival of 5–10-d-old or 20–25-d-old female or male beetles up to 10 d after marking. Based on tethered-flight assays, marking with fluorescent dust generally had no effect on trivial or sustained flight performance of 5–10-d-old and 20–25-d-old females or 15-d-old males. Beetles marked with the oil-soluble dye experienced reductions in trivial flight performance, including reductions in individual flight duration, frequency of flight, and total flight time in a 23-h period, for up to 2 d after marking. Both techniques are efficient, but in comparison with Day-Glo fluorescent powder, beetles marked with Calico oil red retain the mark for a shorter time period. Also, beetles marked with Calico oil red should be provided suitable food for at least 2 d after marking to allow recovery of normal flight behavior before they are used in studies involving flight activity.

KEY WORDS Insecta, *Diabrotica virgifera virgifera*, tethered-flight assay, mark-release-recapture

MASS-MARKING TECHNIQUES are an important element of many insect dispersal studies. The fundamental assumption underlying the use of any marking technique is that it should not unduly influence the survival or behavior of the insect (Southwood 1978). Most studies using marked insects pre-evaluate the influence of the marking agent on longevity and to a more limited degree, reproductive biology (Southwood 1978). However, despite its importance, evaluation of the marking agent on insect behavior, particularly flight behavior, is rare. This evaluation is critical if the movement of marked insects is being used to make inferences regarding rates of dispersal or migration by insects in the field.

A variety of mass-marking techniques have been devised for insects in general (Southwood 1978), and several techniques have been described recently for marking corn rootworm beetles. Lance & Elliott (1990) evaluated the use of an oil-soluble dye incorporated into an artificial diet for mass-marking of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. They evaluated the mark's effect on beetle survival, mark durability, and biases in the detection of marked beetles. Oloumi-Sadeghi & Levine (1990) recently evaluated a mass-marking technique for *D. v. virgifera* using fluorescent powders. They evaluated mark durability and its effect on survival and ovarian development in the field. The influence of these marking techniques on beetle flight behavior has not been rigorously examined. Oloumi-Sadeghi & Levine (1990) observed the behavior of marked *D. v. virgifera* beetles in the field and reported no abnormal effects. However, they did not compare this behavior with behavior of unmarked beetles. Wisniewski (1984) marked the elytra of *D. v. virgifera* with enamel paint and reported that marking had no influence on flight behavior in the laboratory.

Dispersal studies of *D. v. virgifera* require the use of efficient and effective mass-marking techniques, and it is critical that these marking procedures do not unduly influence survival or flight behavior of beetles in the field. This study was done to examine the influence of two mass-marking techniques on survival and flight performance of *D. v. virgifera* in the laboratory. Assays presented here may be useful in evaluating the influences of various marking procedures on other insect species.

Materials and Methods

Adult *D. v. virgifera* beetles (F2) were obtained from a laboratory colony maintained according to established protocols at USDA–ARS, Northern Grain Insects Research Laboratory, Brookings, S. Dak. (Branson et al. 1988). Adults were provided

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an artificial diet (Branson & Jackson 1988) and water.

Marking Techniques. Two different mass-marking techniques were evaluated: a topical fluorescent dust and an internal oil-soluble dye. Day-Glo (Day-Glo Color Corporation, Cleveland) fluorescent dust was applied to beetles in a styrofoam cup that was covered with a plastic lid containing several small holes. The beetles and dust (50 mg per 100 beetles) were agitated for about 15–30 s with a small hand-held air pump, the tip of which was inserted into one of the holes in the lid.

A second marking technique used Calico oil red N-1700 (American Cyanamid, Bound Brook, N.J.), which was milled into a dry artificial diet (Jackson 1986) at a rate of 1% (dry weight) (see Lance & Elliott 1990). Beetles were provisioned with dyed diet and water for 5 d after which time all beetles were well marked. After marking, beetles were confined in an 18-mesh-screen holding cage (0.3 by 0.3 by 0.3 m) and provided dry artificial diet without dye, seedling corn leaves, sweet corn ears, and water.

Evaluation of Mortality. After marking, approximately 50 beetles (5–10 d old) of each sex were held in a screened cage provisioned with the diet described above. The cages were then monitored for mortality 1, 2, 3, 4, 5, and 10 d after marking. All dead beetles were removed and their sex determined. Two cages were established for each marking technique along with two cages of unmarked beetles (controls). The entire experiment was repeated for beetles 20–25 d old. Tests were done at ambient room temperature (about 23°C) with a photoperiod of 14:10 (L:D). Results were analyzed with a G statistic (Sokal & Rohlf 1981) to test for independence of the frequency of control and treatment mortalities. At the end of the experiment beetles were evaluated for mark durability.

Evaluation of Flight Behavior. A computer-interfaced flight device (Wales et al. 1985, Barfield et al. 1988) was used to evaluate the effect of marking on beetle flight behavior. The flight device has been modified slightly from the original design to accommodate the small size of *D. v. virgifera* beetles and to allow the infrared activity detectors to operate in a lighted chamber. Beetles to be flown were anesthetized with carbon dioxide (CO_2) and tethered (on the pronotum) to the end of the flight arm with dental wax (see Wales et al. 1985). Beetles were exposed to CO_2 for <1 min. Beetles were flown for 23 h beginning 1200–1400 hours CST. The device recorded individual flight durations and the number of flights during a 23-h period for each beetle. During flight evaluations, beetles were not fed and remained suspended above the substrate. Flight assays were done in walk-in environmental chambers maintained at 25 ± 1°C and 60 ± 10% RH with a 14:10 photoperiod.

Cohorts of beetles were flight tested at 3 h and at 1, 2, and 5 d after marking. Flight performance of marked beetles was then compared with that of unmarked beetles of a similar age. Flight assays were done for 5–10-d-old and 20–25-d-old females and 15-d-old males for each marking technique, including an unmarked control. Between 31 and 35 beetles were tested for each age group and marking agent. Beetles that died during the 23-h assay period were not included in the analysis. The average duration of individual flights per marked beetle, the number of flights per marked beetle over a 23-h period, and total flight duration per marked beetle were compared with unmarked beetles using the Mann-Whitney rank-sum test (Conover 1980). G tests (Sokal & Rohlf 1981) were used to compare proportions of beetles flying.

Based on previous work (Coats et al. 1986, Naranjo 1990), individual flights by *D. v. virgifera* can be characterized as either trivial or sustained. Based on distinct bimodal distributions of flight duration in western corn rootworm (Naranjo 1990), I define any single flight lasting ≥20 min as a sustained flight and any individual flight <20 min in duration as a trivial flight. These two distinct flight behaviors were analyzed separately.

Results and Discussion

Survival. Marking techniques did not significantly ($P > 0.05$) affect survival of male or female beetles from either age group (Table 1). Survival was >95% even 10 d after marking. Mortality that occurred was evenly distributed throughout this 10-d period. Lance & Elliott (1990) reported somewhat higher rates of mortality for 1–3 wk-old *D. v. virgifera* beetles after marking; however, in most of their tests, they allowed beetles to feed for longer periods on the dyed diet and used a 2% (by weight) concentration of dye. Oloumi-Sadeghi & Levine (1990) found no significant effects on survival of *D. v. virgifera* when using a fluorescent powder (Radiant Color, Richmond, Calif.) applied at a rate of 140 mg per 100 beetles.

Although neither marking agent affected survival, they differed in durability. All beetles dusted with Day-Glo were visibly marked with 10 d if viewed under either an ultraviolet light source or a dissecting microscope. Beetles marked with the oil-soluble dye remained well marked for 5 d but were poorly or not visibly marked after 10 d. Crushing previously marked beetles in acetone and attempting to extract the dye on filter paper did not improve detection.

Flight Behavior. Marking with Day-Glo fluorescent powder caused a significant ($P < 0.05$) increase in flight duration and a reduction in flight frequency but not the total flight time in a 23-h period ($P > 0.05$) for 20–25-d-old females assayed 3 h after marking (Table 2). With this one exception, marking with Day-Glo had no discernible effect on the trivial (Table 2) flight performance of *D. v. virgifera* up to 5 d after marking. In comparison with unmarked beetles, no significant dif-

Table 1. Effect of two marking techniques on mortality of *D. v. virgifera* beetles within 10 d after marking

Age group Sex	% Mortality					
	Fluorescent powder			Internal dye		
	Marked[a]	Control	G^b	Marked[c]	Control	G^b
5–10 d old						
Female	0.00 (104)[d]	0.91 (110)	0.45 NS	1.89 (96)	0.00 (106)	2.05 NS
Male	1.75 (114)	2.80 (107)	0.13 NS	3.00 (100)	1.01 (99)	0.90 NS
20–25 d old						
Female	3.03 (99)	0.00 (114)	1.99 NS	2.68 (112)	3.13 (96)	0.02 NS
Male	3.16 (95)	0.99 (101)	0.53 NS	2.42 (124)	2.88 (139)	0.03 NS

[a] 50 mg of dust per 100 beetles.
[b] G test of independence.
[c] 1% (by weight) concentration in dry artificial diet.
[d] Sample size.

ferences ($P > 0.05$) were detected in the proportion of beetles flying, individual flight times, flight number, or total flight time over a 23-h period. Likewise, marking with fluorescent powder had no apparent effect on sustained flight performance (Table 2). Statistical analyses failed to detect any significant differences ($P > 0.05$) in the proportion of individuals undertaking sustained flight or the length or number of sustained flights. The relative rarity of sustained flight here agrees with results of previous studies (Coats et al. 1986, Naranjo 1990).

In contrast, marking beetles with an internal oil-soluble dye significantly ($P < 0.05$) influenced several aspects of trivial flight performance up to 2 d after marking (Table 3). Individual flight times were reduced in all test groups assayed 3 h after marking, and numbers of flights per beetle were reduced for 3 h, 1 and 2 d, and 3 h after marking for 5–10-d-old and 20–25-d-old females, respectively. Therefore, total flight time over a 23-h period was significantly reduced in beetles from all test groups 3 h after marking and in females 5–10 d old 1 d after marking. The flight performance of young females was affected up to 2 d after mark-

Table 2. Flight performance of *D. v. virgifera* at various times after marking with Day-Glo fluorescent dust, flight durations given in minutes

Age group and sex treatment	Trivial flight					Sustained flight			
	Proportion flying[a]	n	Median: Time per flight per beetle	Median: Flights per beetle	Median: Total flight time per beetle	Proportion flying[a]	n	Median: Time per flight per beetle	Median: Flights per beetle
5–10-d-old females									
3 hour	0.91	31	0.54	54	20.92	0.12	4	25.87	1
1 day	0.89	31	0.34	56	23.88	0.14	5	37.35	1
2 day	0.91	31	0.25	69	20.27	0.14	5	34.23	1
5 day	0.91	32	0.25	68	23.33	0.14	5	23.87	2
Control	0.89	31	0.31	61	22.13	0.17	6	31.67	1
20–25-d-old females									
3 hour	0.68	23	0.52*	22*	6.32	0.00	0	—	—
1 day	0.89	31	0.23	60	14.47	0.06	2	25.96	1
2 day	0.77	24	0.14	34	7.61	0.03	1	41.00	1
5 day	0.85	29	0.23	50	22.13	0.03	1	27.13	1
Control	0.94	30	0.27	50	14.21	0.09	3	45.03	1
10–15-d-old males									
3 hour	0.72	23	0.53	44	25.97	0.09	3	45.83	1
1 day	0.88	28	0.42	57	37.31	0.03	1	20.38	1
2 day	0.84	27	0.46	40	22.27	0.13	4	21.13	1
5 day	0.91	31	0.50	57	31.50	0.09	3	28.52	1
Control	0.91	31	0.45	49	29.23	0.10	3	31.12	1

Asterisks indicate a significant difference in flight performance compared with the control (* significant at $P < 0.05$; Mann-Whitney).
[a] Proportion of beetles flying of 31–35 beetles tested. Flight performance based only on beetles that flew.

Table 3. Flight performance of *D. v. virgifera* at various times after marking by feeding on diet with 1% Calico-red, flight durations given in minutes

Age group and sex treatment	Trivial flight: Proportion flying[a]	n	Median: Time per flight per beetle	Median: Flights per beetle	Total flight time per beetle	Sustained flight: Proportion flying[a]	n	Median: Time per flight per beetle	Median: Flights per beetle
5–10-d-old females									
3 hour	0.83	26	0.14**	40*	3.95**	0.00	0	—	—
1 day	0.85	29	0.49	51*	19.41*	0.06	2	35.40	1
2 day	0.97	34	0.50	39**	24.60	0.17	6	36.62	1
5 day	0.94	32	0.29	55	34.55	0.15	5	40.18	1
Control	0.94	32	0.50	72	31.43	0.24	8	34.46	1
20–25-d-old females									
3 hour	0.73	24	0.11*	26*	4.47*	0.00	0	—	—
1 day	0.79	27	0.41	47	28.34	0.06	2	49.83	1
2 day	0.91	32	0.27	51	23.35	0.11	4	35.58	1.5
5 day	0.82	28	0.29	51	27.12	0.12	4	23.09	1
Control	0.79	27	0.31	43	26.33	0.03	1	45.03	1
10–15-d-old males									
3 hour	0.84	27	0.26*	46	11.45**	0.00	0	—	—
1 day	0.88	30	0.32	63	22.13	0.03	1	23.90	1
2 day	0.97	33	0.37	59	21.98	0.09	3	26.00	2
5 day	0.85	28	0.45	63	28.84	0.09	3	22.48	1
Control	0.97	33	0.45	61	27.92	0.09	3	38.27	1

Asterisks indicate a significant difference in flight performance compared with the control (* significant at $P < 0.05$; ** significant at $P < 0.01$, Mann-Whitney).

[a] Proportion of beetles flying of 31–35 tested. Flight performance based only on beetles that flew.

ing; that of older females and 15-d-old males returned to normal 1 d after marking. Again, no statistical differences ($P > 0.05$) were detected in sustained flight performance between marked and unmarked beetles (Table 3). It is perhaps notable that there was no sustained flight in 5–10-d-old female beetles that were tested 3 h after marking despite the presence of this behavior in control beetles and in beetles tested 3 h after marking with the fluorescent powder. This further suggests that the dye had detrimental effects on overall flight performance.

Various fluorescent powders have been successfully used to mark a variety of insects (Stern & Mueller 1968, Moffitt & Albano 1972, Moth & Barker 1975). They are easy to apply and detect, are fairly durable, and do not cause significant mortality. My results show they caused only minor effects on the flight behavior of older female *D. v. virgifera* beetles 3 h after dusting but no effect on any other group tested. After 1 d, beetles have groomed most of the dust from their bodies; however, grooming does not appear to detract from flight activity.

Calico oil red N-1700 also has been successfully used to mark a variety of insects but with a few minor detrimental effects (Hendricks & Graham 1970, Graham & Mangum 1971, Wilkinson et al. 1972, Lindig et al. 1980). In most cases, the dye is incorporated into the larval diet in low concentrations, and the adults remained visibly marked to the naked eye throughout much of their life. The main advantage of this technique is that insects can be easily marked with minimal handling. Because larvae are reared on seedling corn and not artificial diet, it is more practical to mark *D. v. virgifera* as adults. However, even if adults are offered diet with a fairly high concentration of dye (2%), the accuracy in detecting marked individuals drops with increasing time after marking (Lance & Elliott 1990). My results indicate that at a concentration of 1% the mark is good for only about 5 d.

The flight performance (flight duration, frequency, and total flight time) of young female beetles marked with Calico oil red was significantly altered as long as 2 d after marking. The cause of this behavioral modification is unknown; however, it could be related to a reduction in feeding when the diet is adulterated with the dye. Groups of young male and female beetles given dyed diet gained significantly less weight in a 5-d period than beetles given normal artificial diet (S.E.N., unpublished data). Those given normal and diets with dye gained weight normally but were inconsistently marked suggesting that some beetles tend to avoid the dyed diet. Thus, even though beetles given Calico oil red in their diets did not experience a reduction in survival, reduced feeding was the likely cause of poorer performance in flight assays. Because flight performance improved within several days after marking, beetles were apparently able to recoup when offered a normal diet. At the very least, beetles marked with this technique should be allowed to feed on a suitable diet for 1–2 d before being used in experimental studies involving flight.

In summary, the two mass-marking techniques evaluated were easy to apply and detect but differed in their effects on *D. v. virgifera* flight behavior. Day-Glo fluorescent powder is a highly suitable marking agent because it does not influence either survival or flight behavior in the laboratory. Calico oil red as a diet additive is an efficient marking agent but lacks durability and alters beetle flight performance, which restricts its use for long-term dispersal studies. It should prove effective if beetles are given suitable food for 1–2 d after marking and studies are continued no longer than 3–4 d after release. This time frame makes the mark suitable only for short term studies.

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